

# The Cytoplasmic and Periplasmic Expression Levels and Folding of Organophosphorus Hydrolase Enzyme in *Escherichia coli*

Ali Mohammad Latifi,<sup>1</sup> Khosro Khajeh,<sup>2</sup> Gholamreza Farnoosh,<sup>1,\*</sup> Kazem Hassanpour,<sup>3</sup> and Samaneh Khodi<sup>1</sup>

<sup>1</sup>Applied Biotechnology Research Centre, Baqiyatallah University of Medical Sciences, Tehran, IR Iran

<sup>2</sup>Department of Biochemistry, Faculty of Science, Tarbiat Modares University, Tehran, IR Iran

<sup>3</sup>Medical School, Sabzevar University of Medical Sciences, Sabzevar, IR Iran

\*Corresponding author: Gholamreza Farnoosh, Applied Biotechnology Research Centre, Baqiyatallah University of Medical Sciences, Tehran, IR Iran. Tel/Fax: +98-9155437109, E-mail: zrfarnoosh@yahoo.com

Received 2014 February 1; Revised 2014 June 6; Accepted 2015 July 26.

## Abstract

**Background:** Organophosphorus hydrolase (OPH) is a type of organophosphate-degrading enzyme which is widely used in the bioremediation process.

**Objectives:** In this study, the periplasmic and cytoplasmic productions and the activity of recombinant OPH in *Escherichia coli* were investigated and compared using two pET systems (pET21a and pET26b).

**Materials and Methods:** The sequence encoding the *opd* gene was synthesized and expressed in the form of inclusion body using pET21a-*opd* and in the periplasmic space in pET26b-*opd*.

**Results:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed a band of about 37 kDa with a maximum expression level at 30°C from pET21a-*opd*. However, the obtained results of the periplasmic space extraction of OPH (pET26b-*opd*) showed a very weak band, while the cytoplasmic expression of OPH (pET21a-*opd*) produced a strong protein band.

**Conclusions:** The activities studied by the production of PNP were determined by following the increase at 410 nm. The maximum PNP was produced at 30°C with an optical density of 10.62 in the presence of cytoplasmic expression of OPH (pET21a-*opd*). Consequently, our results suggest cytoplasmic expression system as an appropriate candidate with a high amount of OPH in spite of inclusion body formation, which needs an additional refolding step.

**Keywords:** Organophosphorus, Cytoplasmic, Periplasmic, *Escherichia coli*

## 1. Background

Environmental pollutions caused by organophosphorus compounds (OPs) are one of the major challenges faced by human, due to their widespread application over the past several decades both as chemical war agents and as pesticides, such as parathion, paraoxon, diazinon, chlorpyrifos (Cp), VX, sarin and soman (1, 2). Organophosphorus compounds irreversibly inhibit acetylcholine esterase (AChE), leading to irreparable damage to the nerve cells, resulting in symptoms such as hypotension, bradycardia, bronchoconstriction, and bronchial fluid accumulation, which results in the inability of respiratory muscles to work (3-5). Biodegradation is one of the efficient methods to deal with this fundamental problem (6-10). Organophosphorus hydrolase (OPH) is of great interest enzyme because of its ability to catalyze the hydrolysis of the P-O and P-S bonds in highly toxic organophosphates, which are produced using several approaches in various recombinant systems including multi-gene fusions, fusion with highly-soluble fusion partners, display

on cell surface, and periplasmic secretion (11, 12).

Different systems expressing periplasmic OPH in *Escherichia coli* expression systems have been developed using twin-arginine translocation or *sec* pathway-driven pathways, involving many advantages including simplified downstream processing, enhanced biological activity, conducting in vivo activity assays due to greater access of the targeted protein to the substrate, higher product stability and solubility, N-terminal authenticity of the expressed protein, simpler and cost effective purification due to a lower protein content compared to the cytoplasm, and providing the oxidative environment required for correct protein folding (13-15). However, the low secretion efficiency, incomplete secretion of recombinant protein, insufficient capacity for secretion of overexpressed recombinant protein, the death of host cells, and proteolytic degradation of the product in *E. coli* periplasmic expression systems are problematic despite some successful examples (16). The intracellular produc-

tion of recombinant proteins in *E. coli* cytoplasm which is widely used has several advantages over the secretion of recombinant proteins to the periplasm; however, improper folding of many target proteins may occur during this process.

Improper folding often results in the formation of inclusion bodies despite attempts to optimize the growth condition (17). No significant property was detected to recognize those proteins that have tendency to make inclusion bodies. Correct folding of proteins in cytoplasm can be achieved using several methods which are protein-specific, such as culturing at low temperatures, lowering the protein expression. Although the high protein production in the cytoplasm makes the purification of the soluble target proteins difficult, inclusion bodies have several advantages compared to soluble proteins including the higher accumulation in cytoplasm and increased protein yields as they are proteolysis-resistant, which could be isolated by a simple centrifugation step (15). Accordingly, choosing a suitable strategy to produce a recombinant protein is completely protein-specific.

## 2. Objectives

In this study, we are aimed to investigate the best approach to produce a high-level OPH enzyme with appropriated folding by comparing cytoplasmic and periplasmic expressions of OPH in an *E. coli* host cell.

## 3. Materials and Methods

### 3.1. Design and Optimization of pET21a-*opd* and pET26b-*opd* Construction and Transformation

The sequence encoding the OPH gene from *Pseudomonas diminuta* (Expasy accession No.P0A434) was designed and optimized for *E. coli* by the JCat software (18), Gen Script's ([http://www.genscript.com/cgi-bin/tools/rare\\_codon\\_analysis](http://www.genscript.com/cgi-bin/tools/rare_codon_analysis)) and Mfold (19) software); then, it was synthesized (Biomatik Corp., Canada) into both pET21a and pET26b plasmids. The synthesized sequence, including a 1008-bp fragment with an additional adenine base in the pET26b plasmid, was approved using double-digestion method by 5' *Bam*HI and *Hind*III 3' enzymes. *E. coli* BL21 was transformed using a MicroPulser (Bio-Rad) at 1200V in a 0.1 cm electroporation cuvette. The preparation of electrocompetent cells and electroporation were carried out as described in Bio-Rad MicroPulser electroporation apparatus operating instructions and applications guide (Bio-Rad, #165 - 2100).

### 3.2. Bacterial Strains, Plasmids and Expression

*Escherichia coli* Rosetta-gami cell was used as a host cell for pET21a-*opd* and *E. coli* BL21 was used for pET26b-*opd*. Cells harboring the recombinant plasmids were grown at 37°C in 15 mL LB medium with 20 mg/L kanamycin (sigma, #A1593) for pET26b-*opd* and 80 mg/L ampicillin (sigma,

#A9393) for pET21a-*opd* at 150 rpm. Cells harboring the recombinant plasmids were induced with 0.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) (Sigma, #R1171) after the cells grew to an OD600 = 0.7 and then were cultured for seven hours at 18°C, 30°C and 37°C overnight.

### 3.3. Protein Extraction From the Periplasmic Space

Protein expression was performed based on Khodabakhsh et al. method with a little modification (20). The cultured *E. coli* cells containing recombinant pET26b-*opd* plasmid were centrifuged at 4000  $\times$  g for 20 minutes and the obtained pellet was resuspended in 3750  $\mu$ L of 30mM Tris containing 20% sucrose at pH 8.0, followed by adding 100 mM ethylene diamine tetra acetic acid (EDTA) to a final concentration of 1 mM and incubation on ice for 10 minutes. Cells were then centrifuged at 8000 $\times$ g for 20 minutes at 4°C; then, the supernatant was discarded. The pellet was resuspended in 5 mM MgSO<sub>4</sub>, stirred for 10 minutes in an ice bath, and centrifuged at the previous condition. The supernatant and the pellet were stored at 4°C.

### 3.4. Protein Extraction From the Cytoplasmic Space

For protein extraction, about 25 g of the cells harboring pET21a-*opd* was resuspended in 580 mL of 0.1 mol/L Tris and 20 mmol/L EDTA and homogenized with a shearing rod. Lysozyme (0.25 mg/mL) was added, followed by incubation on ice (30 minutes). Thereafter, centrifugation was carried out for 50 minutes at 4°C (13000 rpm). The pellets were resuspended in 300 mL of 0.1 mol/L Tris, 20 mmol/L EDTA, and 2.5% v/v Triton X-100 and were homogenized. After the centrifugation, the pellets were resuspended in 300 mL buffer containing 0.1 mol/L Tris, 20 mol/L EDTA, and 0.5% v/v Triton X-100 and were homogenized. The samples were then centrifuged (30 minutes, 4°C, 13000 rpm), and the pellets were resuspended in 250 mL of 0.1 mol/L Tris and 20 mmol/L EDTA. The prepared inclusion body was stored at 20°C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% running gel using the method of Laemmli (21) and the protein bands were visualized by staining with Coomassie Brilliant Blue R250 (Merck, Darmstadt, Germany).

### 3.5. Activity Assay

The OPH activity assay was based on a method developed by Tang et al. (22). For each assay, 200  $\mu$ L of the produced OPH in a solution fractions was added to 700  $\mu$ L of 75 mM Tris-HCl buffer with 50 M CoCl<sub>2</sub> (pH 8.0) and 100  $\mu$ L of 20 mM paraoxon in 20% methanol. The reaction was conducted in a 1.5-mL plastic tube for 10 minutes at 37°C; 200  $\mu$ L of the reaction medium was added to a 96-deep-well plate and the production of PNP was determined by following the increase at 410 nm ( $\epsilon_{410}$  = 16500 M<sup>-1</sup> cm<sup>-1</sup>) and one unit of OPH activity was defined as mmol PNP produced per minute. The calculation method was described by Bisswanger (23).

## 4. Results

### 4.1. Plasmid Sequencing

The molecular lengths of *opd* in pET21a and pET26b were 1008 bp and 1009 bp, respectively. The released fragments from pET21a-*opd* and pET26b-*opd* digestion by *Bam*HI and *Hind*III restriction enzymes approved the successful cloning of the *opd* gene. The plasmid sequencing was performed for further confirmation of correct cloning (Figure 1).

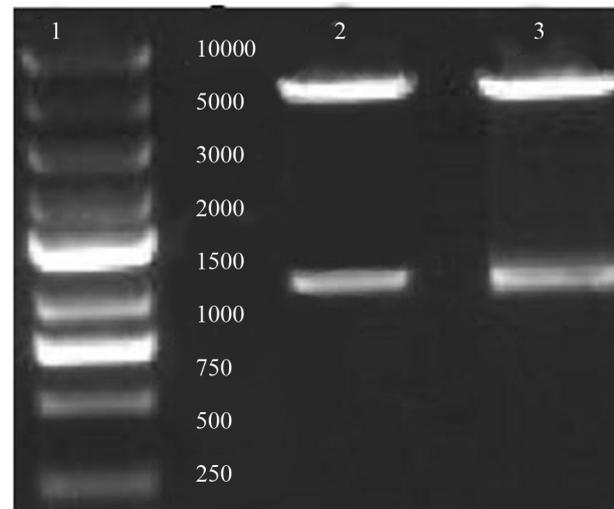
### 4.2. Protein Expression Analysis by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The obtained results of cytoplasmic and periplasmic extractions of soluble and inclusion body fractions from both constructions using SDS-PAGE showed a band of about 37 kD (Figure 2). The maximum expression level was observed in fractions obtained at 30°C. The results revealed an increased expression level in both fractions in the produced OPH by the pET21a-*opd* construct compared to that of the pET26b, which showed high-level production and appropriated folding.

### 4.3. Activity Assay

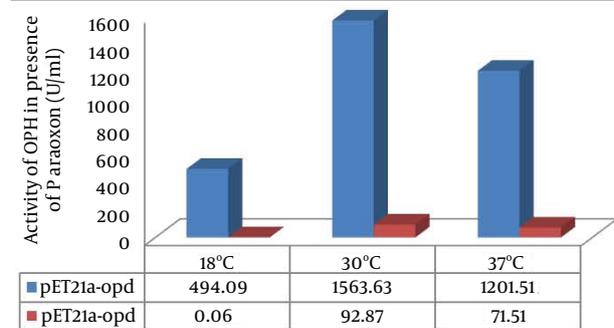
The change in the PNP absorbance in the reaction solution is shown in Figure 3. By investigating the activity of the produced OPH in the solution fractions against paraoxon, most of the activity was expressed in soluble samples from the pET21a-*opd* construction (Figure 3). This increase in the activity was predictable due to the strong bands in the SDS-PAGE (Figure 2). The highest level of activity was observed in the produced OPH at 30°C with maximum activity of 1563.63 U/mL in the presence of the soluble fractions obtained from pET21a-*opd* and the lowest level of activity was at 18°C in the presence of the soluble fractions from pET26b-*opd*.

**Figure 1.** Double-Digestion of Recombinant Plasmids



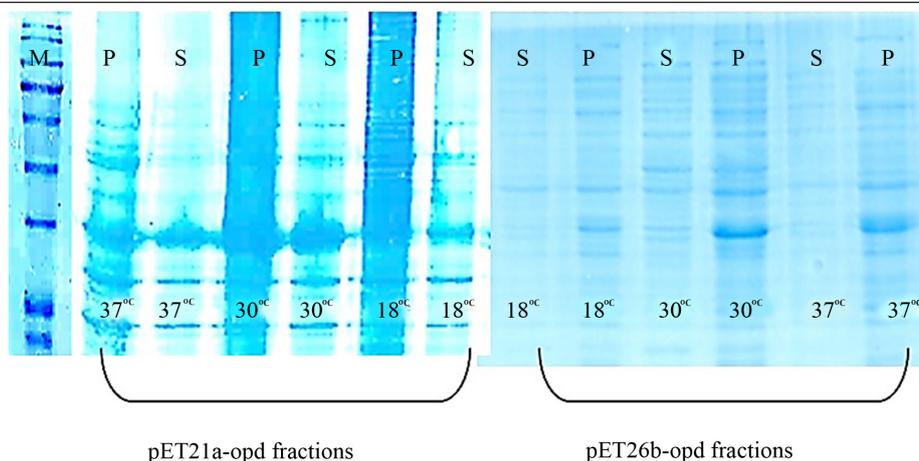
Lane 1, standard molecular weight marker; lane 2, pET21a-*opd* double digestion; lane 3, pET26b-*opd* double digestion.

**Figure 3.** The Activity Assay of Soluble Fractions of pET21a-*opd* and pET26b-*opd* Enzymes



Comparing the activity at three temperatures, the highest activity was observed at 30°C and the lowest at 18°C.

**Figure 2.** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Analysis of pET21a-*opd* and pET26b-*opd* Soluble and Insoluble Fractions



The fractions were expressed in *E. coli* Rosetta-gami cells with 0.5 mM IPTG for seven hours at 18°C, 30°C and 37°C. M, molecular weight marker; P, pellet; S, soluble.

## 5. Discussion

In this study, cytoplasmic and periplasmic extractions of the expression of recombinant OPH protein in *E. coli* were investigated and compared using the pET system. pET26b bearing the N-terminal PelB signal sequence was applied for potential periplasmic localization (24). The pelB leader sequence consists of 22 N-terminal amino acid residues which can be attached to any other protein (on the DNA level), resulting in a transfer of such fused protein to the periplasmic space of Gram-negative bacteria such as *E. coli*, where the sequence is removed by a signal peptidase (25). pET26b vector with an N-terminal His-tag and an IPTG-inducible T7 promoter was used for the intracellular expression in *E. coli*. The obtained results of protein extraction from both plasmids using SDS-PAGE showed a band about 37 kD.

The periplasmic secretion of *E. coli* contains several advantages over intracellular production such as separation from cytoplasmic proteins, enhanced biological activity, enhanced product solubility and the ease of protein purification (26). Moreover, it is accepted that proteins with disulfide bonds could only be properly folded in the periplasm due to the existence of alkaline phosphatase, which is active only in the periplasm (27). In a study by Tang et al. methyl parathion hydrolase (MPH) was targeted to the periplasm by fusing MPH to the twin-arginine signal peptide of trimethylamine N-oxide reductase (TorA), which showed only 35% activity in the periplasm fraction and indicated that this barrier of substrate transport could be eliminated by periplasmic secretion. Although, in a study by Kang et al. the Tat-driven periplasmic secretion of OPH was presented as a potential strategy to overcome traditional substrate diffusion limitations in whole cell biocatalyst systems for detoxification of organophosphate compound (28).

The reason was the fact that in the Tat pathway, proteins are at least partially folded prior to export, without the requirement of ATP. The Tat pathway has been successfully used for periplasmic secretion of several foreign proteins such as cofactor-containing, multimeric, and disulfide-containing proteins (28). Of course, the production of many proteins such as pancreatic prekallikrein, human epidermal growth factor, protease inhibitors and t-PA in the periplasm of *E. coli* BL21 have shown low concentrations, misfolding and consequently inactivation in the primary trials. However, the periplasmic space extraction of pET26b plasmid showed a very weak band, which is triggered by the fact that misfolded proteins in the periplasm rapidly are degraded through stress-related degradation pathway of misfolded proteins in the periplasm by DegP protease (27, 29). Consequently, SDS-PAGE analysis and activity assay results demonstrated that cytoplasmic expression system is efficient for the production of OPH protein in prokaryotic systems; however, high amount of OPH was obtained in the form of inclusion bodies.

Cytoplasmic production also involved several advan-

tages such as no need for outer-membrane disruption to recover target proteins and avoiding intracellular proteolysis by periplasmic proteases, leading to continuous recombinant proteins production (29). In our study, the fractions obtained by pET26b showed over-expression of the OPH protein in inclusion body forms with a weak band on SDS-PAGE for the soluble fraction. Such form is mostly composed of recombinant proteins, is protected from proteolytic degradation, and can be easily isolated from the cell debris. In addition, lower temperature significantly increases the total amount of protein due to the slower growth, which provides enough time for protein folding and reduces the rate of cell lysis and the proteolytic activity of the proteases. Conclusively, recombinant protein targeting can be performed at any compartments of cytoplasm, periplasm and the extracellular milieu. Considering the balance between advantages and disadvantages of each compartment based on the properties of the target protein is needed to determine the proper one for targeting recombinant proteins (15). Based on our results, no considerable protein production was detected in the periplasmic OPH fraction and the cytoplasmic expression of OPH showed a more efficient system for high-level expression and appropriate folding, in spite of inclusion body formation; therefore, an additional refolding step is eligible.

## Acknowledgments

We thank the manager and staff of Applied Biotechnology Research Centre, Baqiyatallah University of Medical Sciences, Tehran, Iran, for useful discussions and critical reading of the manuscript. It should be noted that this paper was extracted from a research project in Applied Biotechnology Research Center of Baqiyatallah University of Medical Sciences. This paper was extracted from a Ph.D. thesis.

## Footnotes

**Authors' Contribution:** Study concept and design: Gholamreza Farnoosh, Ali Mohammad Latifi, Khosro Khajeh; acquisition of data: Samaneh Khodi; analysis and interpretation of data: Gholamreza Farnoosh and Hossein Aghamollaei; drafting of the manuscript: Kazem Hassanpour and Gholamreza Farnoosh; critical revision of the manuscript for important intellectual content: Gholamreza Farnoosh and Khosro Khajeh.

**Funding/Support:** This study was financially supported by grant from Applied Biotechnology Research Centre, Baqiyatallah University of Medical Sciences, Tehran, Iran.

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